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# STUDIES ON VARIANTS OF POLIOMYELITIS VIRUS*

## I. Experimental Segregation and Properties of Avirulent Variants of Three Immunologic Types‡

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The search for spontaneously occurring or experimentally developed avirulent strains of poliomyelitis virus suitable for human immunization calls for fundamental knowledge of the conditions which favor the appearance and segregation of variants or mutants, as well as of the properties by which they may be characterized. Previous demonstrations of modifications of the paralytogenic activity of poliomyelitis virus in monkeys have depended on continued propagation in tissues of another host. Theiler (1) was the first to report that, after 50 rapid intracerebral passages in mice, the Lansing strain no longer produced signs of poliomyelitis in 8 intracerebrally inoculated *rhesus* monkeys. In other laboratories (2, 3) the same strain of virus still produced paralytic poliomyelitis in *rhesus* monkeys after approximately 200 or more intracerebral passages in mice. Other strains of poliomyelitis virus have also been found to lose much, although not all, of their paralytogenic activity in monkeys after passage in mice, cotton rats, or hamsters, while gaining in virulence for the new hosts (4–7). When it was shown that cultivation of the Type 1, Brunhilde strain in human non-nervous tissue resulted in a marked reduction of its paralytogenic activity in monkeys (8), the question arose whether the effect was due to propagation in the tissues of another host or to propagation in non-nervous tissue. Accordingly in the design of the present studies it was decided to keep the host constant and to vary the conditions of cultivation. Viruses possessing a high original virulence for the *cynomolgus* monkey by various routes were selected for study. The purpose of the present communication is to present data which indicate that serial propagation in *cynomolgus*

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kidney tissue cultures had no effect on virulence for *cynomolgus* monkeys when single or small numbers of virus particles were used to initiate the cultures, while rapid passages with large inocula led to the appearance of avirulent variants, which ultimately were separated from the virulent variants by the terminal dilution technique.

## *Materials and Methods*

*Viruses—Type 1 Poliomyelitis.*—The Mahoney strain, obtained from Dr. Thomas Francis, Jr., of the University of Michigan School of Public Health, had the following history prior to its use in this laboratory. A virus, recovered from a pool of stools from 3 Mahoney children had 6 intracerebral passages in *rhesus* monkeys, followed by 3 subcutaneous passages in *cynomolgus* monkeys and one intracerebral passage in *rhesus* monkeys. The virus had 2 serial intracerebral passages in *cynomolgus* monkeys in this laboratory: the first with 1 ml. of a 20 per cent suspension in 2 monkeys, and the second with 1 ml. of a 2 per cent suspension in 101 monkeys. The spinal cords and medullas from the 101 monkeys, killed on the 1st day of paralysis, were made up into one large pool which was frozen in a chest containing dry ice. Aliquots of this pool were used in all experiments with this virus.

*Type 2 Poliomyelitis.*—The Y-SK strain was obtained as 10th mouse passage material from Dr. Joseph L. Melnick of the Yale University School of Medicine. After 4 additional passages in mice (virus being harvested only from paralyzed animals), this strain received 2 serial, intracerebral passages in *cynomolgus* monkeys: the first with 0.5 ml. of 10 per cent suspension of mouse CNS in 23 monkeys, and the second with 0.5 ml. of 10 per cent suspension of *cynomolgus* CNS in 94 monkeys. The spinal cords and medullas of the 94 monkeys, killed on the 1st day of paralysis, were made up into one large pool which was frozen in a chest containing dry ice. Aliquots of this pool were used in all experiments with this virus.

*Type 3 Poliomyelitis.*—After somewhat more than 20 intracerebral passages in *rhesus* monkeys, the Leon strain had received 8 serial passages in tissue cultures of *rhesus* testis before it was sent to this laboratory by Dr. Joseph L. Melnick. Subsequent passages in this laboratory were in *cynomolgus* kidney tissue cultures which will be described later.

*Monkeys Used, Procedures of Infection, and Observations.*—*Cynomolgus* monkeys from the Philippine Islands were used in all the tests. Intracerebral injections placed the inoculum in the thalamic region, subcutaneous injections in the midaxillary line of the chest wall, and intramuscular injections in the left or right leg muscles. Infection by the oral route was carried out by a standard procedure which was found to yield optimum results. The virus was fed, 1 hour after food, by placing a rubber-tipped syringe on the teeth and allowing the monkeys to swallow 5 ml. of the expressed fluid. This dose of virus was given once a day for 3 days. Temperatures of the monkeys were not taken because earlier studies indicated that they provided no significant data. The monkeys were observed for paralysis for a period of 4 to 5 weeks. All monkeys which showed no obvious manifestations of poliomyelitis or which died without exhibiting such manifestations were submitted to an extensive histologic examination of the central nervous system. 20 to 40 levels of the spinal cord, 5 levels of medulla pons, at least one level of midbrain through the superior colliculi, as well as sections through the thalamus and hypothalamus were examined as a routine. Care was taken to include the roots in the sections of the spinal cord, as a guide both to the age of lesions and the number of neurones affected. In all monkeys which received virus by mouth, the olfactory bulbs were examined in semiserial sections as well as 1 or more sections through the anterior perforated substance. Any monkey exhibiting evidence of neuronal lesions in the olfactory pathway was eliminated from the experiment. Blood for antibody studies was obtained from the femoral vessels before infection and from the heart at the termination of the experiment.

*Tissue Cultures.*—Roller tubes containing either minced kidney or testis from *cynomolgus* monkeys were used as indicated in the experiments. Tissue from monkeys weighing not more

than 3 to 4 pounds gave the best results. The thoroughly washed, minced tissue was suspended in chick embryo extract and placed in a portion of the tube smeared with a cotton swab moistened in chicken plasma. At first medium 199 was used, and the fluids were changed every 4 days. However, this was soon replaced by a medium consisting of lactalbumin hydrolysate, Simms's ox serum ultrafiltrate, and Earle's solution. (Each 100 ml. of medium contains 10 ml. of 5 per cent lactalbumin hydrolysate adjusted to pH 8.5, 22.5 ml. of the ultrafiltrate, and 67.5 ml. of complete Earle's solution; penicillin and streptomycin added to final concentration of 100 units and 0.1 mg. respectively per ml.) Each roller tube containing 2 ml. of this medium receives an inoculum of 0.2 ml. It has not been necessary to change the fluid in these tubes. The kidney tissue cultures were maintained in a roller drum, while testis tissue cultures yielded entirely satisfactory results in stationary racks. Only the cortex of the kidney was used and an adequate outgrowth of epithelial cells was usually obtained in 6 to 7 days. More recently crystalline, bovine albumin in a final concentration of 0.1 per cent was incorporated in the medium for kidney tissue cultures, because the yield of good tubes seems to have been increased thereby. Trypsin inhibitor is purposely excluded from the medium to permit the epithelial cells to grow directly on the glass of the roller tubes. After adequate outgrowth has occurred the tubes remain serviceable for another 7 to 14 days without any change of medium. The larger volumes of virus required for virulence tests by different routes were grown in 250 ml. centrifuge bottles. The entire inner surface of these bottles, coated with chicken plasma, is covered with a large number of pieces of minced tissue. 40 ml. of medium, containing trypsin inhibitor in a final concentration of 0.05 mg. per ml. but not bovine albumin, was added to each bottle. The bottles were maintained in a roller drum.

When proper care was taken to dissect away all of the tunica from the testis, only fibroblasts grew out in the plasma clot. Adequate growth was usually present in 3 to 4 days, and the tubes remained serviceable for another 2 to 3 weeks without change of medium.

*Tests for Poliomyelitis Antibody in Tissue Culture.*—Only kidney tissue cultures were used for this purpose, because the virus preparations used yielded a 10-fold or higher titer in kidney than in testis cultures, and also because the results are more clean cut and easier to read. Screening tests were performed by mixing undiluted serum with enough diluted virus tissue culture fluid to yield 100 $TCD_{50}$ (50 per cent tissue culture doses) per 0.2 ml. Sometimes, as will be indicated in the text, smaller or larger amounts of virus were used. The mixtures were left at room temperature (about 22 to 25°C.) for 1 hour and then distributed in 0.2 ml. amounts to each of 3 tubes. In quantitative tests for antibody 10-fold dilutions of serum are used. The tubes were then put back in the roller drum for at least another day. Comparative tests showed that the outcome was not affected if the tubes were then left in stationary racks. The tubes were examined 1 day after inoculation to check on toxicity of the serum but this has not been encountered with the monkey sera stored in the frozen state. Although the result is frequently apparent at 3 days, final readings were made at 4 and 8 days. In the screening test, antibody was recorded as being present when at least 2 of the 3 tubes showed no cytopathogenic effect at 8 days. When the 4 day reading indicated neutralization, while the 8 day reading did not, the result was regarded as suggestive of a minimal amount of antibody. In the quantitative tests the results, in tubes containing a constant amount of virus and different dilutions of serum, were of a nature which permitted the calculation of a 50 per cent end-point by the method of Reed and Muench.

### *Experiments with Type 1 Poliomyelitis Virus (Mahoney Strain)*

*Observations on Propagation of the Virus in Kidney Tissue Culture.*—The rapidity with which different concentrations of this strain of virus produced a cytopathogenic effect in tissue cultures of different passage levels is recorded in Table I.

The earliest cytopathogenic change, consisting of disintegration of intercellular syncytium and the characteristic rounding up of individual epithelial cells, affects small groups of cells which stand out as plaques in a sheet of otherwise normal appearing epithelial cells. All the epithelial cells are, as a rule, similarly affected within 24 hours. It is of interest, therefore, to note the "zone phenomenon" in the culture tubes inoculated with the original virus in the form of a centrifuged extract of *cynomolgus* spinal cord and medulla. Not only was the cytopathogenic effect in the tubes inoculated with the 10 per cent suspension delayed for several days, but approximately 50 per cent of the epithelial cells remained unaffected for a period of at least 5 days after the initial cytopathogenic change was observed. This "zone phenomenon" is not due to the concentration of tissue extract, because it does not occur with all viruses

TABLE I

*Serial Propagation of Type 1 Poliomyelitis Virus (Mahoney Strain) in cynomolgus Kidney Tissue Culture Employing Progeny of Single or Small Numbers of Virus Particles*

| Dilution of CNS suspension or tissue culture fluid | Day on which 50 per cent or greater cytopathogenic change was observed in tubes at indicated passage level | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 31* | 32 |
| $10^{-1}$ | 5, 6, 7‡ | 1, 1 | | | | | | | | | |
| $10^{-2}$ | 2, 2, 2 | 1, 1 | | | | | | | | | |
| $10^{-3}$ | 2, 2, 2 | 2, 2 | 2, 2 | 1, 1 | | | | | 2, 2 | 1, 1 | |
| $10^{-4}$ | 2, 3, 4 | 2, 2 | 2, 2 | 1, 2 | 3, 3 / 5 | 2, 2 | 2, 2 | 2, 2 | 2, 3 | 1, 1 | 3,3 |
| $10^{-5}$ | 5, 5§, 0 / 5 | 2, 3 / 6 | 3, 3 / 4 | 3, 4 | 0, 0 | 3, 4 | 3, 3, 3 | 7, 0, 0 / 8 | 4, 0 / 6 | 2, 2, 2, 2, 2 | 2, 4 |
| $10^{-6}$ | 0, 0, 0 | 0, 0 | 7, 0 | 3, 0 / 5 | 0, 0, 0 | 5, 0, 0, 0 / 6 | 4, 0, 0, 0 / 7 | 0, 0, 0, 0 | 0, 0, 0, 0 | 3, 3, 3, 3, 4 | 3,‖0 / 6 |
| $10^{-7}$ | 0, 0, 0 | | 0, 0 | 0, 0 | 0, 0, 0 | 0, 0, 0, 0 | 0, 0, 0, 0 | 0, 0, 0 | 0, 0 | 4, 4, 5, 0, 0 / 8 | 0, 0 |
| $10^{-8}$ | | | | | | | | | | 0, 0, 0, 0, 0 | 0, 0 |

* The culture fluid for this titration was derived from large bottles seeded with a large inoculum of virus.

‡ There was not only a delay in appearance of the cytopathogenic change in these tubes, but approximately 50 per cent of the outgrowing renal epithelial cells remained unaffected.

§ The fluid from the underlined tubes was used for the next passage; the number in the denominator refers to the day after inoculation when the culture fluid was harvested.

‖ The fluid from this tube was used to inoculate the large bottles which yielded the kidney passage 33 virus employed in the tests for virulence and immunogenic activity.

The interfering variant or substance was eliminated in the very next passage, initiated with the progeny from the terminal dilution tubes.

In the first 5 serial passages, no attempt was made to subculture from single tubes of the terminal dilution. Whether or not the subsequent passages from single tubes represent cultivation from the progeny of single particles is problematical, even if one accepts the statistical evidence of Dulbecco and Vogt (9) that a single plaque is formed, as a rule, by a single virus particle. It is of interest to note, however, the extent to which 1 or 2 tissue culture doses had multiplied by 24 hours or less after the appearance of extensive cytopathogenic change. The culture fluid from the terminal dilution of passage 1, harvested on the day the first extensive cytopathogenic change was observed, contained $10^{5.5}$ $TCD_{50}$ per 0.2 ml. or $10^{6.5}$ $TCD_{50}$ in the 2 ml. of fluid (not counting the tissue or cells) present in the tube.

After the first 9 serial, terminal dilution passages, in which subcultures were made with the progeny of single or small numbers of virus particles, a large amount of virus representing kidney passage 10 was grown in 250 ml. centrifuge bottles in which 40 ml. of medium was

TABLE II

*Effect of Different Methods of Propagation of Type 1 Poliomyelitis Virus (Mahoney Strain) in cynomolgus Kidney Tissue Cultures on (a) Intracerebral Virulence for cynomolgus Monkeys and (b) Cytopathogenic Titer in Cultures of cynomolgus Renal Epithelium and Testicular Fibroblasts*

| Dilution of CNS suspension or culture fluid inoculated | Paralytic score, incubation periods and lesions in monkeys, and titers in tissue cultures inoculated with indicated materials | | | |
|---|---|---|---|---|
| | A<br>*cynomolgus* CNS suspension | B<br>A + 9 terminal dilution passages + 1 large inoculum kidney passage 10 | C<br>B + 20 rapid passages with large inocula kidney passage 30 | D<br>C + 2 terminal dilution passages + 1 large inoculum kidney passage 33 |
| Undiluted | — | 5/5 (5, 5, 5, 5, 6) | 1/4 (17,* 0, 0, 0) | 0/4 (0, 0, 0, 0) |
| $10^{-1}$ | — | 5/5 (4, 6, 6, 6, 8) | 2/4 (10, 11, 0, 0) | 0/4 (0, 0, 0, 0) |
| $10^{-2}$ | — | 5/5 (5, 5, 6, 8, 15) | 3/4 (12, 13, 16, 0) | 0/4 (0, 0, 0, 0) |
| $10^{-3}$ | 9/10 {6, 6, 7, 7, 8, 10 / 11, 13, 15, NP} | 5/5 (5, 5, 7, 7, 9) | 2/4 (9, 13, 0, 0) | 0/4 (0, 0, 0, 0) |
| $10^{-4}$ | 5/10 {9, 9, 10, 11, 24 / 0, 0, 0, 0, 0} | 3/5 (5, 6, 9, 0, 0) | 1/9 (13,‡ 0, 0, 0) / (0, 0, 0, 0, 0) | 0/4 (0, 0, 0, 0) |
| $10^{-5}$ | 1/10 {6, 0, 0, 0, 0 / 0, 0, 0, 0, 0} | 4/5 (8, 8, 12, 20, 0) | 0/4 (NP, 0, 0, 0) | 0/4 (0, 0, 0, 0) |
| $10^{-6}$ | 1/10 {8, 0, 0, 0, 0 / 0, 0, 0, 0, 0} | 1/4 (9, 0, 0, 0, ⑧) | 0/4 (0, 0, 0, 0) | 0/4 (0, 0, 0, 0) |
| $10^{-7}$ | 0/9 {0, 0, 0, 0, 0 / 0, 0, 0, 0} | — | — | — |
| 50 per cent paralytic end-point... | $10^{4.1}$ | $10^{5.3}$ | Irregular to $10^{-4}$ | 0 |
| $TCD_{50}$/ml. or gm. in renal epithelium..... | $10^{6.0}$ | $10^{5.7}$ | $10^{7.0}$ | $10^{7.2}$ |
| $TCD_{50}$/ml. or gm. in testis fibroblasts..... | $10^{2.2}$ | $10^{4.2}$ | $10^{7.2}$ | $10^{6.2}$ |

Legends: 0 = no lesions found in CNS; NP = no paralysis but lesions present in CNS; ⑧ = dead 8th day, but no lesions.

* Spinal cord of this monkey contained $10^{7}$ $TCD_{50}$/gm., but intracerebral passage yielded this result: 1/4 (10, 0, 0, 0).

‡ Spinal cord of this monkey contained $10^{4.2}$ $TCD_{50}$/gm., but intracerebral passage yielded this result: 3/4 (5, 12, 18, 0).

inoculated with 0.1 ml. of undiluted tissue culture fluid from the $10^{-5}$ terminal dilution tube of passage 9. A pool of culture fluids collected 4 and 8 days respectively after introduction of the virus was used in the virulence tests.

The 11th kidney passage began with the undiluted tissue culture fluid of the large pool described above, and subsequent passages up to 29 were made every 24 hours by subinoculating 0.2 ml. of the undiluted tissue culture fluid. At the time of each passage all the outgrowing epithelial cells exhibited the characteristic cytopathogenic change and $10^5$ to $10^6$ $TCD_{50}$

were probably transferred at each passage. A large amount of virus representing kidney passage 30 was again prepared in 250 ml. centrifuge bottles by inoculating 40 ml. of medium with 0.1 ml. of undiluted fluid from passage 29. The pool of the two harvests collected at 3 and 6 days had a titer of $10^{7.9}$ $TCD_{50}$ per ml.

The large lot of kidney passage 33 virus was prepared in large bottles by inoculating 40 ml. of medium with 0.1 ml. of undiluted fluid from the $10^{-6}$ terminal dilution tube of passage 32 (see Table I). The pool of the two harvests made at 3 and 6 days had a titer of $10^{7.2}$ $TCD_{50}$ per ml.

*Tests for Virulence by Intracerebral Route.*—The data in Table II show the differences in virulence exhibited by the Mahoney strain of virus propagated in different ways.

The development and incidence of paralysis, the occurrence of CNS lesions in monkeys without obvious manifestations, the incubation period in paralyzed animals are all correlated with another standard of reference; *i.e.*, concentration of virus as measured by the cytopathogenic effect in tissue cultures *in vitro*. Antibody response was not used as a routine test here, because it was found that antibodies do not develop as quickly in *cynomolgus* monkeys infected with Mahoney virus by intracerebral inoculation as in those infected by peripheral routes. The results indicate that the virus contained in the CNS suspension was either more readily, or certainly as readily, detected by the *in vitro* test in kidney tissue cultures as by intracerebral injection. It is evident that after 10 passages in kidney cultures, using the progeny of single or small numbers of particles, the virus remained fully virulent, the minimal infective dose being practically identical by intracerebral inoculation and by cytopathogenic effect in kidney tissue cultures.

After 20 daily passages with large inocula, the kidney tissue culture virus exhibited a very marked diminution in intracerebral virulence despite an approximately 100-fold increase in titer as measured by its cytopathogenic effect on renal epithelial cells *in vitro*. The following modifications are to be noted: (*a*) a certain proportion of monkeys receiving as much as 80 million $TCD_{50}$ of virus not only remained well but also failed to exhibit any CNS lesions; (*b*) the incubation period in the paralyzed monkeys was 2 to 3 times as long and the disease was no longer fatal (Table V); and (*c*) paralysis was rare (1/9) in monkeys inoculated with 8,000 $TCD_{50}$ and absent in those receiving 800 $TCD_{50}$ or less.

The data, taken together with the results of passage on 2 of the monkeys (see Table II), strongly suggested that the culture fluid might contain a mixed population of virulent and avirulent virus particles. The complete absence of paralysis and CNS lesions in the 28 monkeys inoculated intracerebrally with the kidney passage 33 culture fluid, obtained after 2 terminal dilution purifications, provides support for this interpretation. The establishment in subsequent tests that the virus in this culture fluid is antigenically Type 1 poliomyelitis virus warrants the conclusion that a new variant or mutant, which is avirulent by the intracerebral route in *cynomolgus* monkeys, appeared during the course of the rapid passages in *cynomolgus* kidney cultures and sufficiently outgrew the ancestral virulent virus to permit its segregation by the terminal dilution technique.

*Tests for Virulence by the Subcutaneous or Intramuscular Routes.*—The relationship between the capacity to infect, as measured by development of antibody, and the capacity to produce paralysis or lesions in the nervous system,

provides a quantitative index to the virulence of the virus by the subcutaneous or intramuscular routes. The preinoculation and postinoculation sera were always tested for antibody simultaneously, and only a distinct change from negative to positive was regarded as evidence of infection. The data shown in Table III, compared with those in Table II, indicate that minimal amounts of the virus contained in the original CNS suspension produced paralysis and infection more readily by the subcutaneous route than by the intracerebral route. It is also evident that the minimal amount of virus required to produce a cytopathogenic effect in renal epithelial cells *in vitro* and infection by the subcutaneous route is about the same. It should also be noted that an occasional monkey becomes infected without exhibiting either paralysis or CNS lesions regardless of the amount of virus that is inoculated. In another test, not shown in Table III, 5 *cynomolgus* monkeys, which received 1 ml. of the $10^{-3}$ dilution of the same lot of virus subcutaneously, for some unknown reason all developed antibody without exhibiting either paralysis or CNS lesions.

While the results obtained with the kidney passage 10 tissue culture fluid are irregular as regards development of paralysis and CNS lesions, it is nevertheless evident that minimal amounts of virus produced both paralysis and infection. The subsequent tests were performed by injecting the virus into the leg muscles of one side in the hope that it might indicate the extent to which the virus invaded along the regional nerves from the inoculated site. The different behavior of the virus in the kidney passage 30 tissue culture fluid is as striking after intramuscular injection as it was in the intracerebrally inoculated monkeys. Only 8 of the 40 monkeys exhibited paralysis, which with one exception (see Table V) was mild or limited. Thus the one monkey in the $10^{-5}$ group which developed paralysis had only a unilateral facial paralysis and this was also true of 1 of the 2 paralyzed monkeys in the $10^{-2}$ group. It is also noteworthy that the CNS lesions in the non-paralyzed monkey in the $10^{-5}$ group were limited to one facial nucleus and one vagal nucleus in the medulla with only focal lesions in the cervical spinal cord and none in many levels of the lumbar cord, while in the non-paralyzed monkey which received the undiluted virus the lesions were limited to the lumbar cord. These data, together with the observations that in all the other monkeys paralysis began either in the inoculated extremity or both lower extremities, suggest that in some monkeys the virus invaded along the regional nerves from the inoculated muscles while in others some other pathway leading to the medulla may have been used. Attention should also be drawn to the fact that a certain proportion of monkeys inoculated with 80,000 $TCD_{50}$ or less failed to become infected as judged by development of antibody.

None of the monkeys inoculated with kidney passage 33 tissue culture fluid exhibited paralysis. However, 2 monkeys were found dead 8 and 9 days after inoculation; one of these showed no CNS lesions while the other showed scattered lesions the extent of which could not be evaluated because of the advanced stage of tissue decomposition which occurred on a hot August night. The one monkey in the group inoculated with undiluted tissue culture fluid ($10^{7.2}$ $TCD_{50}$) which showed CNS lesions had a very limited type of involvement; only 4 of 19 levels of the spinal cord (2 lumbar and 2 thoracic) showed focal infiltrative lesions occupying areas of only a few neurones in the anterior horns; at one level of lumbar cord a root lesion affecting only a few fibers was present; no lesions were found above the spinal cord. One of the monkeys inoculated with the $10^{-3}$ dilution had to be sacrificed 21 days after inoculation because it had been badly bitten by a cage mate. Acute poliomyelitis lesions were found in all levels of the spinal cord, extensive in some regions and only focal in others, while only minimal lesions were found in the medulla and none in the hypothalamus and thalamus. A suspen-

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## TABLE III

*Effect of Different Methods of Propagation of Type 1 Poliomyelitis Virus (Mahoney Strain) in cynomolgus Kidney Tissue Cultures on Paralytogenic and Infective Capacity in Subcutaneously or Intramuscularly Inoculated cynomolgus Monkeys*

| Dilution of CNS suspension or culture fluid inoculated | A (subcutaneous) *cynomolgus* CNS suspension | | | B (subcutaneous) A + 9 terminal dilution passages + 1 large inoculum kidney passage 10 | | | C (intramuscular) B + 20 rapid passages with large inocula kidney passage 30 | | | D(intramuscular) C + 2 terminal dilution passages + 1 large inoculum kidney passage 33 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Paralysis | Incubation period or CNS lesions | Total* infection | Paralysis | Incubation period or CNS lesions | Total infection | Paralysis | Incubation period or CNS lesions | Total infection | Paralysis | Incubation period or CNS lesions | Total infection |
| Undiluted | — | — | — | 4/5 | 4, 4, 6, 8, 0 | 5/5 | 3/5 | 8, 9, 10, NP, 0 | 5/5 | 0/5 | NP, 0, 0, 0, 0 | 5/5 |
| $10^{-1}$ | 4/5 | 8, 8, 9, 10, 0 | 5/5 | 3/5 | 6, 7, 12, NP, NP | 5/5 | 2/5 | 9, 14, 0, 0, 0 | 5/5 | 0/5 | 0, 0, 0, 0, ($D^9$-P)‡ | 5/5 |
| $10^{-2}$ | 5/5 | 6, 7, 7, 8, 0 | 5/5 | 1/5 | 15, 0, 0, 0, 0 | 5/5 | 2/5 | 10, 16, 0, 0, 0 | 5/5 | 0/5 | 0, 0, 0, 0, 0 | 3/5 |
| $10^{-3}$ | 4/5 | 7, 8, 8, 9, 8 | 5/5 | 3/5 | 11, 13, 17, 0, 0 | 5/5 | 0/5 | 0, 0, 0, 0, 0 | 3/5 | 0/5 | 0, 0, 0, NP,§ ($D_8$-0) | 1/4 |
| $10^{-4}$ | 4/5 | 9, 12, 16, 20, 0 | 5/5 | 2/5 | 12, 18, 0, 0, 0 | 3/5 | 0/5 | 0, 0, 0, 0, 0 | 3/5 | 0/5 | 0, 0, 0, 0, 0 | 1/5 |
| $10^{-5}$ | 3/4 | 7, 8, 14, 0 | 4/4 | 2/5 | 9, 12, 0, 0, 0 | 4/5 | 1/5 | 10, NP, 0, 0, 0 | 2/5 | 0/4 | 0, 0, 0, 0, ② | 0/4 |
| $10^{-6}$ | 0/4 | 0, 0, 0, 0, ⑪ | 1/4 | 5/5 | 9, 10, 11, 11, 12 | 5/5 | 0/5 | 0, 0, 0, 0, 0 | 2/5 | 0/5 | 0, 0, 0, 0, 0 | 1/5 |
| $10^{-7}$ | — | — | — | — | — | — | 0/5 | 0, 0, 0, 0, 0 | 0/5 | 0/5 | 0, 0, 0, 0, 0 | 0/5 |
| 50 per cent end-point | $10^{4.7}$ | | $10^{5.7}$ | Irregular 20/35 | | | Irregular 8/40 | | Irregular to $10^{-6}$ | 0 | | |
| $TCD_{50}$ per ml. or gm. in renal epithelium | $10^{6.0}$ | | | $10^{5.7}$ | | | $10^{7.9}$ | | | $10^{7.2}$ | | |

Legends same as for Table II.

* Total infection includes monkeys which developed antibody without exhibiting paralysis or CNS lesions.

‡ This monkey died without exhibiting any obvious manifestations; poliomyelitis lesions present—minimal above spinal cord but extent of involvement of spinal cord difficult to evaluate because of marked postmortem autolysis.

§ This monkey was killed 21 days after inoculation, because it was badly bitten by cage mate. Focal acute poliomyelitis lesions found. $10^{4.7}$ $TCD_{50}$/gm. of spinal cord and medulla but intracerebral passage to 4 *cynomolgus* monkeys yielded this result: 0/4 (NP-focal, 0, 0, 0).

sion of spinal cord and medulla of this monkey was titered in kidney tissue culture and passaged intracerebrally in 4 *cynomolgus* monkeys to determine whether the lesions resulted from the multiplication of a virulent variety of the virus. Although the suspension had a titer of $10^{4.7}$ $TCD_{50}$/gm. in kidney tissue culture, none of the 4 passage monkeys exhibited any ob-

TABLE IV

*Manifestations and Lesions in Various Parts of Body in cynomolgus Monkeys Inoculated Intramuscularly with Avirulent Variant of Type 1 Poliomyelitis Virus (Mahoney, Kidney Passage 33)*

| Dilution of culture fluid inoculated | *Cynomolgus* No. | Manifestations | Lesions in various parts of body | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | CNS | Inoculated muscles | Kidneys | Testes or ovaries | Pancreas | Adrenals | Liver | Spleen | Heart |
| Undiluted | 1 | 0 | 0 | ? | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $10^{-2}$ $TCD_{50}$ | 2 | 0 | 0 | " | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 0 | 0 | " | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 4 | 0 | 0 | " | 0† | 0 | 0 | 0 | 0 | 0 | 0 |
| | 5 | 0 | 0 | " | 0† | 0 | 0 | 0 | 0 | 0 | 0 |
| $10^{-3}$ | 6 | 0 | 0 | | | | | | | | |
| $10^{-4.2}$ $TCD_{50}$ | 7 | 0 | 0 | | | | | | | | |
| | 8 | 0 | 0 | | | | | | | | |
| | 9 | 0 | 0 | | | | | | | | |
| | 10 | 0 | 0 | | | | | | | | |

? = sections through 4 to 5 levels of the inoculated leg muscles revealed not more than 1 to 3 very minute foci of infiltration with mononuclear cells.

† = one of the kidneys exhibited several very small foci of intertubular infiltration with mononuclear cells, which are of doubtful, if any, significance in relation to the inoculated virus.

TABLE V

*Modification of Severity of Paralytic Disease Resulting from Inoculation of Type 1 Poliomyelitis Virus (Mahoney Strain) after Many Rapid Passages with Large Inocula in cynomolgus Kidney Tissue Cultures*

| Route of inoculation | Material inoculated | No. of paralyzed monkeys observed | Prostrate or dead | |
|---|---|---|---|---|
| | | | No. | Per cent |
| Intracerebral | *Cynomolgus* CNS suspension | 14 | 12 | 86 |
| | *Cynomolgus* kidney passage 10* | 23 | 23 | 100 |
| | *Cynomolgus* kidney passage 30‡ | 8 | 1 | 13 |
| Subcutaneous or intramuscular | *Cynomolgus* CNS suspension | 20 | 20 | 100 |
| | *Cynomolgus* kidney passage 10* | 19 | 18 | 95 |
| | *Cynomolgus* kidney passage 30‡ | 7 | 1 | 14 |

* Progeny of single or small numbers of virus particles in 9 preceding serial passages.

‡ Progeny of large numbers of virus particles after 20 rapid serial passages.

vious signs of infection of the CNS; 3 of these 4 monkeys revealed no CNS lesions and the 4th exhibited focal lesions in some levels of the spinal cord, medulla, midbrain and thalmus; the root lesions which were found in this monkey indicated that the process was at least 10 to 14 days old and that only a few neurones were affected.

Since lesions were found in 3 of the 38 intramuscularly inoculated monkeys but in none of the 28 intracerebrally inoculated animals, it appeared desirable to inoculate additional monkeys intramuscularly to determine how often one could obtain evidence of invasion of

the spinal cord by this seemingly avirulent virus. The absence of paralysis and CNS lesions in a test on 10 monkeys, shown in Table IV, indicates that invasion of the spinal cord occurs only rarely and the possibility now being considered and tested is that it may depend on the accidental inoculation of one of the large nerves which are distributed among the leg muscles. If it should prove possible to produce lesions regularly by direct inoculation into nerve trunks or the spinal cord, an explanation would still be required for the absence of such lesions after direct intracerebral injection of virus or for the negative intracerebral passage result with virus recovered from the spinal cord. This intriguing problem will be discussed further after the results of similar tests with the other types of poliomyelitis virus are analyzed.

Table IV also shows the negative results obtained in the histologic examination of the viscera of 5 *cynomolgus* monkeys which were inoculated intramuscularly with $10^{7.2}$ $TCD_{50}$ of virus. This was done to determine whether or not the new variant, which exerted so marked a cytopathogenic effect on epithelial cells and fibroblasts growing out of *cynomolgus* kidneys and testes respectively *in vitro*, would produce any demonstrable lesions in various tissues of *cynomolgus* monkeys *in vivo*. The inoculated leg muscles were sectioned at 4 to 5 levels in order not to miss the site in which the 1 ml. of the tissue culture fluid was injected. Although very minute foci of infiltration with mononuclear cells, occupying areas not exceeding the size of 1 to 3 muscle fibers, were found in each of the monkeys, similar foci were found in monkeys inoculated with a $10^{-6}$ dilution of a culture fluid which failed to produce antibody. Occasional small foci of infiltration in the kidneys of 2 of the monkeys and in the portal spaces of the liver of most monkeys were not regarded as significant because they are so often encountered in control monkeys. Although these histologic examinations were made about 1 month after inoculation, one may conclude, nevertheless, that the variant contained in the kidney passage 33 tissue culture fluid does not produce any significant, demonstrable residual lesions in the muscles or viscera of *cynomolgus* monkeys. It should also be noted here that while 1 $TCD_{50}$ of virus contained in the original CNS suspension was capable of initiating infection in subcutaneously inoculated monkeys, most of the monkeys inoculated with 1000 $TCD_{50}$ or less of the kidney passage 33 virus failed to develop antibody (Table III). The antibody tests recorded in Table III were all performed against 500 $TCD_{50}$ of kidney passage 5 virus, and when they were repeated with 50 $TCD_{50}$ all but one of the monkeys inoculated with the $10^{-2}$ or $10^{-3}$ dilutions became positive, while only 1 additional monkey of those which received smaller amounts of virus became positive.

*Tests for Virulence by Oral Route.*—Infection by the oral route with the virus contained in the *cynomolgus* CNS suspension either produced paralysis, as a rule of the prostrating or fatal type, or antibody without CNS lesions.

Among 200 monkeys fed the $10^{-1}$ suspension, focal poliomyelitis lesions in the spinal cord and medulla were encountered only once in a monkey without obvious signs of paralysis. A remarkably constant relationship was observed between the number of monkeys which developed paralysis and those which developed antibody without manifest or histologic evidence of involvement of the CNS. Regardless of the dilution of virus suspension that was fed to the monkeys, 2 paralytic infections occurred for each inapparent infection. This also held in the group of monkeys which ingested the kidney passage 10 tissue culture fluid, but not in the group which ingested even larger amounts of the kidney passage 33 virus. Although the total incidence of infection was less than that in monkeys fed smaller amounts of the virulent virus, 8 of a group of 20 monkeys developed antibody while none developed paralysis (Table VI).

The entire group of 20 monkeys was challenged 5 weeks later by being fed the virulent virus contained in the original CNS suspension. The results, shown in Table VII, indicate that 13 (68 per cent) of the 19 control monkeys, which were fed simultaneously and had no

antibody to begin with, developed paralysis, while the 6 remaining monkeys developed antibody; the 2:1 relationship between paralytic and inapparent infection may again be noted here. In the group which had previously ingested avirulent virus, the results were different in the 8 which had antibody prior to challenge and the 12 which did not, suggesting that little if any immunity developed in the absence of demonstrable antibody. On the other hand, antibody levels as low as 1:3 to 1:10 (final dilution of serum) sufficed to protect against paralysis, although probably not against a limited reinfection. The latter assumption is based on the antibody response after challenge with the virulent virus. It is noteworthy that normal monkeys, without spontaneously occurring antibody, generally develop high titers of antibody as a result of primary inapparent infection with virulent virus (see data for controls in Table VII). Inapparent infection with the avirulent virus produced significantly lower titers in

TABLE VI

*Effect of Different Methods of Propagation of Type 1 Poliomyelitis Virus (Mahoney Strain) in cynomolgus Kidney Tissue Cultures on Virulence by Oral Route in cynomolgus Monkeys*

| Material tested | Log $TCD_{50}$ per gm. or ml. | Effect of feeding indicated dilution of virus | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Undiluted | | $10^{-1}$ | | $10^{-2}$ | | $10^{-3}$ | | $10^{-4}$ | | $10^{-5}$ | |
| | | Paralysis | Infection* | Paralysis | Infection | Paralysis | Infection | Paralysis | Infection | Paralysis | Infection | Paralysis | Infection |
| *Cynomolgus* CNS suspension | 6.0 | — | — | 12/20 | 18/20 | 11/18 | 16/18 | 5/20 | 7/20 | 2/18 | 3/18 | 0/17 | 0/16 |
| *Cynomolgus* kidney passage 10 Progeny of 9 terminal dilution passages | 5.7 | 12/19 | 18/19 | | | | | | | | | | |
| | | 1:2 | | | | | | | | | | | |
| *Cynomolgus* kidney passage 33 Progeny of terminal dilution after 20 rapid passages | 7.2 | 0/20 | 8/20 | | | | | | | | | | |

*Note.*—Only monkeys without histologic evidence of involvement of the olfactory pathway are included in this analysis. There were no monkeys with lesions in the CNS without paralysis.

* This includes monkeys which developed antibody without exhibiting either paralysis or CNS lesions.

approximately 50 per cent of the monkeys. After challenge with the virulent virus, the monkeys which had titers of 1:10 or less, to begin with, exhibited a 4- to 18-fold rise, while those with titers of 1:32 or more showed no such rise in antibody. Since there is no evidence that mere ingestion of virus could produce a booster effect, it appears highly probable that the virus multiplied in the monkeys with low titers of antibody.

*Effect of Different Methods of Propagation on the Cytopathogenic Effect on Fibroblasts.*—The original 10 per cent suspension, which exhibited a zone phenomenon in renal epithelial cells (Table I), showed no such zone in fibroblasts growing out of *cynomolgus* testis, but no effect was observed in higher dilutions prepared from the original suspension. Even subculture from the first set of testis tubes yielded no cytopathogenic effect beyond the $10^{-1}$ dilution. If this limited cytopathogenic activity of the original virus suspension were due to the fact that the virus particles possessing this property were

genetically distinct and present in only small numbers, they should have been eliminated by the terminal dilution passages. This did not happen, however, since the kidney passage 10 virus possessed a higher titer of fibroblastic cytopathogenic activity, even though the effect was delayed for as long as 7 to 14 days. The further rapid passages in kidney tissue cultures resulted in a marked enhancement of cytopathogenic activity for testicular fibroblasts, not only as

TABLE VII

*Resistance and Antibody Response of cynomolgus Monkeys Which Ingested Avirulent Virus (Mahoney, Kidney Passage 33) to Subsequent Challenge with Unmodified Parent Virus by Oral Route*

| Group | Antibody prior to challenge with virulent virus | Monkey No. | Result of challenge | | Level of antibody at indicated periods | | |
|---|---|---|---|---|---|---|---|
| | | | Paralysis (incubation period) | Lesions | Before virus | After avirulent virus | After virulent virus |
| Avirulent virus by mouth $10^{7.6}$ $TCD_{50}$ once a day on Sept. 3, 4, 5, 1953 | Positive | 1 | 0 | 0 | 0 | 1:3 | 1:32 |
| | | 2 | 0 | 0 | 0 | 1:5 | 1:20 |
| | | 3 | 0 | 0 | 0 | 1:5 | 1:20 |
| | | 4 | 0 | 0 | 0 | 1:10 | 1:180 |
| Virulent virus by mouth $10^{5.7}$ $TCD_{50}$ once a day on Oct. 8, 9, 10, 1953 | | 5 | 0 | 0 | 0 | 1:32 | 1:56 |
| | | 6 | 0 | 0 | 0 | 1:100 | 1:180 |
| | | 7 | 0 | 0 | 0 | 1:180 | 1:320 |
| | | 8 | 0 | 0 | 0 | 1:180 | 1:180 |
| | Negative | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 2 | 0 | 0 | 0 | 0 | 0 |
| | | 3 | 0 | 0 | 0 | 0 | 1:32 |
| | | 4 | 0 | 0 | 0 | 0 | 1:320 |
| | | 5 | 0 | 0 | 0 | 0 | 1:320 |
| | | 6 | 0 | 0 | 0 | 0 | 1:320 |
| | | 7–12 | 9, 10, 10, 10, 10, 15 | + | 0 | 0 | not tested |
| Controls | Positive | 1 | 0 | 0 | 1:3 | — | 1:10 |
| Virulent virus by mouth $10^{5.7}$ $TCD_{50}$ once a day on Oct. 8, 9, 10, 1953 | Negative | 1 | 0 | 0 | 0 | — | 1:10 |
| | | 2 | 0 | 0 | 0 | — | 1:180 |
| | | 3 | 0 | 0 | 0 | — | 1:320 |
| | | 4 | 0 | 0 | 0 | — | 1:320+? |
| | | 5 | 0 | 0 | 0 | — | 1:560 |
| | | 6 | 0 | 0 | 0 | — | 1:560 |
| | | 7–19 | 7, 9, 9, 9, 9, 10, 10 10, 10, 10, 10, 11, 15 | + | 0 | — | not tested |

regards titer but also as regards rapidity of effect which was observed within 1 to 2 days with the higher concentrations of virus.

*Effect of Intraspinal Injection in Mice.*—In view of the report by Li and Schaeffer (6) that the Mahoney strain of virus, after a number of passages in monkey testis cultures, produced transmissible paralysis in intraspinally inoculated mice, several groups of mice were inoculated with virus at different stages of cultivation. Kidney passage 5 and kidney passage 30 culture fluids were each injected intraspinally in 30 mice without effect. It is of special interest, therefore, that the kidney passage 33 culture fluid produced paralysis in al-

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most all intraspinally inoculated mice, and the virus recovered from their spinal cords was neutralized by Type 1 but not by Type 2 or Type 3 poliomyelitis antisera.

### *Experiments with Type 2 Poliomyelitis Virus (Y-SK Strain)*

*Observations on Propagation of Virus in Kidney Tissue Culture.*—The work with this strain of virus actually preceded that done with the Type 1 virus. The two strains behaved very similarly in kidney tissue cultures and the observations previously recorded for the Mahoney strain also apply here. It

TABLE VIII

*Serial Propagation of Type 2 Poliomyelitis Virus (Y-SK Strain) in cynomolgus Kidney Tissue Culture Employing Progeny of Single or Small Numbers of Virus Particles*

| Dilution of CNS suspension or tissue culture fluid | Day on which 50 per cent or greater cytopathogenic change was observed in tubes at indicated passage level | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 50* |
| $10^{-1}$ | 2, 3 | 1, 1 | 1, 1 | | | | | | | |
| $10^{-2}$ | 2, 2 | 1, 1 | 1, 1 | | | | | | | |
| $10^{-3}$ | 2, 2 | 2, 2 | 2, 2 | 1, 1 | 1, 1 | 2, 2 | 1, 1 | 1, 1 | | 1, 2 |
| $10^{-4}$ | $\frac{3, 3}{4}$ | 3, 3 | 2, 2 | 1, 2 | 2, 2 | 2, 2 | 2, 2 | 2, 2 | 2, 3 | 2, 2 |
| $10^{-5}$ | 5, 6 | $\frac{4}{4}$, 8, 0 | $\frac{3, 3}{4}$ | $\frac{3, 3}{5}$ | 3, 3 | $\frac{3}{4}$, 5 | $\frac{2, 2}{4}$ | 2, 2 | $\frac{3, 3}{4}$ | 2, 2, 2 |
| $10^{-6}$ | 0, 0 | 0, 0, 0 | 0, 0 | 0, 0 | $\frac{3}{4}$, 0 | 0, 0 | 0, 0 | 4, 0 | 0, 0, 0 | $\frac{2}{2}$,‡ 4, 8 |
| $10^{-7}$ | 0, 0 | 0, 0 | 0, 0 | 0, 0 | 0, 0 | 0, 0 | 0, 0 | $\frac{4}{4}$, 0, 0 | 0, 0, 0 | 0, 0, 0 |

Legends same as in Table I.

* The culture fluid for this titration was derived from 2 tubes of kidney passage 49 harvested 18 hours after inoculation with undiluted fluid from the previous passage.

‡ 0.1 ml. of undiluted fluid from this tube was used to inoculate 40 ml. of culture fluid in large bottles for kidney passage 51, which yielded avirulent virus in tests on monkeys.

should be noted, however, that no "zone phenomenon" was obtained with the 10 per cent *cynomolgus* CNS suspension of this strain of virus (Table VIII).

After the first 9 terminal dilution passages (see Table VIII) a large amount of virus was grown in many roller tubes, each of which contained 4 ml. of medium 199 and 0.1 ml. of the $10^{-1}$ dilution of the culture fluid from the $10^{-5}$ tubes of kidney passage 9. The tubes were harvested twice at 3-day intervals, and a pool of the 2 harvests was used for the virulence tests.

The progeny of a $10^{-6}$ terminal dilution tube of kidney passage 10 was used as undiluted tissue culture fluid to start the next series of passages. 0.2 ml. of undiluted culture fluid was transferred to 2 ml. of medium at each passage. The cytopathogenic effect was almost complete at 24 hours, but transfers were made *every 48 hours*. A large amount of virus representing kidney passage 30 was prepared in 250 ml. centrifuge bottles by inoculating 40 ml. of the lactalbumin hydrolysate medium with 0.1 ml. of undiluted fluid from passage 29. Two harvests were made at 4 day intervals, the first having a titer of $10^{7.8}$ $TCD_{50}$ per ml. and the second $10^{6.5}$ $TCD_{50}$ per ml. Three terminal dilution passages were made beginning with the progeny of virus in a $10^{-7}$ dilution tube of the pool of the 2 harvests of kidney passage 30, and each subsequent passage was also made from a single $10^{-7}$ dilution tube. The large amount

of virus, representing kidney passage 34, was prepared by inoculating 40 ml. of medium in large bottles with 0.1 ml. of undiluted culture fluid from the $10^{-7}$ tube in kidney passage 33. A pool of 2 harvests, made at 3 day intervals, had a titer of $10^{7.9}$ $TCD_{50}$ per ml. and was used for the virulence tests.

When the virulence tests showed that the 3 terminal dilution purifications failed to yield an avirulent variant, it was decided to go back to kidney passage 30 and start another series of rapid passages with large inocula but this time make the transfers every 24 hours or less, instead of every 48 hours. 0.2 ml. of undiluted culture fluid was transferred every 24 hours up to passage 39, and after that the transfers were made every 16 to 18 hours up to passage 49. The titration of the fluid harvested at 18 hours from kidney passage 49, shown in Table VIII, indicates that $10^{7.2}$ $TCD_{50}$ of virus per ml. were already present in the tube. The large amount of virus representing kidney passage 51, used in the virulence tests, was prepared by inoculating 0.1 ml. of undiluted fluid from one $10^{-6}$ dilution tube into 40 ml. of medium in large bottles. A pool of 2 harvests, at 3-day intervals, yielded $10^{6.9}$ $TCD_{50}$ of virus per ml.

*Tests for Virulence by Intracerebral Route.*—The virus contained in the original *cynomolgus* CNS suspension was detected as readily, and perhaps somewhat more readily, by its cytopathogenic effect on *cynomolgus* renal epithelial cells as by intracerebral injection in *cynomolgus* monkeys (Table IX). The same is true of the virus contained in the kidney passage 10 culture fluid, although here again it should be noted that the cytopathogenic titer in kidney tissue culture is 8 times higher than the intracerebral titer. The pattern of response in the test with the kidney passage 30 culture fluid (large inocula transferred every 48 hours) is quite different from that obtained with the kidney passage 30 culture fluid of the Mahoney strain (large inocula transferred every 24 hours) in that (*a*) there is regular involvement of the nervous system, with or without apparent paralysis, all the way to the end-point, and (*b*) there is no significant prolongation of the incubation period (compare data in Tables IX and II). Modification of the virulence of the virus, nevertheless, became evident in 2 ways: (*a*) a marked increase in the proportion of monkeys exhibiting focal or limited CNS lesions without paralysis (8 of 25 as compared with 1 of 33 in the previous 2 tests), and (*b*) a marked increase in the proportion of monkeys with paralysis whose disease did not develop to the prostrating or fatal stage (see Table XI). If one counts all monkeys with CNS lesions, the 50 per cent end-point for the amount of "CNS-pathogenic" virus per ml. of culture is $10^{4.6}$ as compared to the titer of $10^{7.5}$ obtained in kidney tissue culture. This 800-fold difference in titer may be compared with the 4-fold difference observed in the test with the original CNS suspension and the 8-fold difference in the test with the kidney passage 10 culture fluid. If this 800-fold difference represented an excess of virus particles which are incapable of producing CNS lesions after intracerebral injection one would have expected that the 3 terminal dilution passages, with progeny from single $10^{-7}$ dilution tubes, would have yielded a variant that was avirulent for *cynomolgus* monkeys by the intracerebral route; but the test with kidney passage 34 culture fluid indicates that this was not achieved. It would appear, therefore, that in the kidney passage 30 culture fluid the population of virus particles

was either homogenous, each particle possessing a diminished capacity for propagating or producing damage in the CNS of *cynomolgus* monkeys, or that virulent and avirulent particles were present in approximately equal numbers, which would make it difficult to separate them by the terminal dilution technique. It was on the latter assumption that the passages were speeded up to 24 and then to 16 to 18 hours, in the hope that the avirulent particles, if such

TABLE IX

*Effect of Different Methods of Propagation of Type 2 Poliomyelitis Virus (Y-SK Strain) in cynomolgus Kidney Tissue Cultures on (a) Intracerebral Virulence for cynomolgus Monkeys, (b) Cytopathogenic Titer in Cultures of cynomolgus Renal Epithelium and Testicular Fibroblasts, (c) Intracerebral Titer in Mice*

| Dilution of CNS suspension or culture fluid inoculated | Paralytic score, incubation periods, and lesions in monkeys, and titers in tissue cultures and mice inoculated with indicated materials | | | | |
|---|---|---|---|---|---|
| | A<br>*Cynomolgus* CNS suspension (2 ml.) | B<br>A + 9 terminal dilution pass. + 1 large inoculum<br>kidney passage 10 | C<br>B + 20 passages every 48 hrs. with large inocula<br>kidney passage 30 | D<br>C + 3 terminal dilution passages + 1 large inoculum<br>kidney passage 34 | E<br>C + 19 rapid passages + 1 terminal dilution + 1 large inoculum<br>kidney passage 51 |
| Undiluted | — | 4/4 (5, 7, 7, 7) | 5/5 ( 4, 7, 8, 8, 12) | 4/4 ( 5, 5, 7, 21) | 0/4 (0, 0, 0, 0) |
| $10^{-1}$ | — | 4/4 (4, 6, 6, 7) | 4/5 ( 4, 8, 8, 12, NP) | 3/4 ( 4, 6, 12, NP) | 0/4 (0, 0, 0, 0) |
| $10^{-2}$ | — | 4/4 (6, 8, 12, 13) | 2/5 ( 8, 12, NP?, NP?, NP?) | 3/4 ( 6, 7, 21, NP) | 0/4 (0, 0, 0, 0) |
| $10^{-3}$ | — | 3/4 (4, 16, 17, NP) | 3/4 ( 7, 14, 15, NP, ⓓ) | 4/4 (10, 12, 13, 21) | 0/4 (0, 0, 0, 0) |
| $10^{-4}$ | 4/4 ( 3, 5, 7, 8) | 4/4 (8, 9, 9, 13) | 2/5 ( 7, 13, NP, NP, NP) | 1/4 ( 9, NP, 0, 0) | 0/4 (0, 0, 0, 0) |
| $10^{-5}$ | 4/4 ( 7, 8, 9, 28) | 2/3 (9, 10, 0, ⓓ) | 1/5 (16, 0, 0, 0, 0) | 3/4 ( 8, 9, 24, 0) | 0/4 (0, 0, 0, 0) |
| $10^{-6}$ | 2/6 ( 8, 8, 0, 0, 0, 0) | 0/3 (0, 0, 0, ⓓ) | 0/3 ( 0, 0, 0) | 0/4 ( 0, 0, 0, 0) | 0/4 (0, 0, 0, 0) |
| $10^{-7}$ | 1/6 (10, 0, 0, 0, 0, 0) | 0/4 (0, 0, 0, 0) | — | — | — |
| $10^{-8}$ | 0/3 ( 0, 0, 0) | — | — | — | — |
| 50 per cent end-point per gm. or ml. — Paralysis | $10^{6.6}$ | $10^{6.8}$ | Irregular to $10^{-5}$ | Irregular to $10^{-5}$ | 0 |
| 50 per cent end-point per gm. or ml. — Renal epithelium | $10^{6.2}$ | $10^{6.2}$ | $10^{7.8}$ | $10^{7.9}$ | $10^{6.9}$ |
| 50 per cent end-point per gm. or ml. — Testicular fibroblasts | $<10^{1}$ | $10^{2?}$ | $10^{6.2}$ | — | $10^{6.2}$ |
| 50 per cent end-point per gm. or ml. — Mice | $10^{8.3}$ | $10^{1.5}$ | 1 or less | 1 or less | 1 or less |

Legends same as Table II.
NP? = focal lesions in brainstem but none in spinal cord.

were present, might multiply more rapidly and overgrow the virulent virus sufficiently to permit separation by the terminal dilution technique. Whether or not that is how it happened, the kidney passage 51 tissue culture fluid, with a renal cytopathogenic titer of $10^{6.9}$ per ml., produced neither paralysis nor lesions in any of the 28 monkeys used in the test. This variant was proved to be a Type 2 poliomyelitis virus.

*Tests for Virulence by Subcutaneous or Intramuscular Routes.*—The virus in

the original *cynomolgus* CNS suspension as well as in kidney passage 10 culture fluid possessed the capacity for producing paralysis in the majority of subcutaneously inoculated monkeys; an occasional monkey developed CNS lesions without paralysis and the remainder all developed antibody without any demonstrable CNS lesions (Table X). The paralytogenic capacity of minimal amounts of virus by the subcutaneous route was not determined. The olfactory bulbs and anterior perforated substance were examined in most monkeys, because of the demonstration some years ago with other strains of poliomyelitis virus that intravenously injected virus may follow the olfactory pathway into the CNS (10–12) but no lesions were found.

TABLE X

*Effect of Different Methods of Propagation of Type 2 Poliomyelitis Virus (Y-SK Strain) in cynomolgus Kidney Tissue Cultures on Paralytogenic and Infective Capacity in Subcutaneously or Intramuscularly Inoculated cynomolgus Monkeys*

| Dilution of CNS suspension or culture fluid inoculated | A (subcutaneous) *Cynomolgus* CNS suspension ($10^{6.2}$ $TCD_{50}$/gm.) | B (subcutaneous) Kidney passage 10* ($10^{6.2}$ $TCD_{50}$/ml.) | C (intramuscular) Kidney passage 30* ($10^{7.5}$ $TCD_{50}$/ml.) | D (intramuscular) Kidney passage 34* ($10^{7.9}$ $TCD_{50}$/ml.) | | | E (intramuscular) Kidney passage 51* ($10^{6.9}$ $TCD_{50}$/ml.) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day of paralysis or CNS lesions | Day of paralysis or CNS lesions | Day of paralysis or CNS lesions | Paralysis | Incubation period or CNS lesions | Total infection | Paralysis | Incubation period or CNS lesions | Total infection |
| Undiluted | | 7, 7, 7, 8, 8 8, NP, 0, 0, 0 | 5, 5, 6, 6, 9 NP, NP, 0, 0, 0 | 4/5 | 4, 6, 6, 6, NP | 5/5 | 0/5 | NP, 0, 0, 0, 0 | 5/5 |
| $10^{-1}$ | 7, 7, 7, 8, 8 9, 10, 11, NP, 0 | | | 4/5 | 6, 6, 6, 7, NP | 5/5 | 0/5 | NP, 0, 0, 0, 0 | 5/5 |
| $10^{-2}$ | | | | 2/5 | 6, 9, NP, 0, 0 | 5/5 | 0/5 | 0, 0, 0, 0, 0 | 4/5 |
| $10^{-3}$ | | | | 2/5 | 6, 8, NP, 0, 0 | 5/5 | 0/5 | 0, 0, 0, 0, 0 | 3/5 |
| $10^{-4}$ | | | | 0/5 | 0, 0, 0, 0, 0 | 5/5 | 0/5 | Tested for active immunity | 1/5 |
| $10^{-5}$ | | | | 0/5 | 0, 0, 0, 0, 0 | 3/5 | 0/5 | | 0/5 |
| $10^{-6}$ | | | | 0/5 | 0, 0, 0, 0, (D) | 2/5 | 0/5 | | 0/5 |
| $10^{-7}$ | | | | 0/5 | 0, 0, 0, 0, 0 | 1/5 | 0/5 | | 0/5 |

* See Table IX for details regarding these passages.

In conformity with the evidence of modification of virulence observed in the intracerebrally inoculated animals, it may be noted that the incidence of prostrating or fatal disease was lower among the monkeys inoculated with kidney passage 30 or kidney passage 34 culture fluid than among those inoculated with the original CNS suspension or kidney passage 10 culture fluid (Table XI). It is, furthermore, evident that neither paralysis nor CNS lesions appeared in any of the 20 monkeys inoculated with 8 to 8,000 $TCD_{50}$ of the virus in kidney passage 34 culture fluid, although antibody developed in 5 of 5 inoculated with 8,000 $TCD_{50}$ and even in 1 of 5 receiving only 8 $TCD_{50}$ (Table X). In further conformity with the results of the intracerebral test for virulence, none of the 40 monkeys inoculated intramuscularly with various amounts of the kidney passage 51 culture fluid developed paralysis. The

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CNS of the 20 monkeys which received $10^{6.9}$ to $10^{8.9}$ $TCD_{50}$ were examined histologically 33 to 34 days after inoculation, while the remaining 20 monkeys which received smaller amounts of virus, and, with one exception, failed to develop demonstrable antibody by 46 days after inoculation, were tested for active immunity by an intramuscular injection of a 5 per cent suspension of the original CNS virus. There was no evidence of active immunity in the absence of antibody, the incidence of paralysis being about the same in all groups as in 10 controls which were inoculated at the same time.

As was the case with the Mahoney virus, 2 of the 20 intramuscularly inoculated monkeys exhibited CNS lesions while none was found in any of the intracerebrally inoculated monkeys which received the same amounts of the Y-SK, kidney passage 51 culture fluid. In one of these 2 monkeys the process was focal and limited to the lumbar cord, where it was extensive

TABLE XI

*Modification in Severity of Paralytic Disease Resulting from Inoculation of Type 2 Poliomyelitis Virus (Y-SK Strain) after Propagation in Different Ways in cynomolgus Kidney Tissue Cultures*

| Route of inoculation | Material inoculated | No. of paralyzed monkeys observed | Prostrate or dead | |
|---|---|---|---|---|
| | | | No. | Per cent |
| Intracerebral | CNS suspension | 11 | 11 | 100 |
| | Kidney passage 10* | 21 | 17 | 81 |
| | Kidney passage 30* | 17 | 5 | 29 |
| | Kidney passage 34* | 18 | 7 | 39 |
| Subcutaneous or intramuscular | CNS suspension | 8 | 5 | 63 |
| | Kidney passage 10* | 6 | 4 | 67 |
| | Kidney passage 30* | 5 | 2 | 40 |
| | Kidney passage 34* | 14 | 3 | 21 |

* Procedures of cultivation described in previous tables and text.

at one level in one anterior horn, and minimal at other levels; many sections through the thoracic and cervical cord, medulla, midbrain, thalamus, and hypothalamus revealed no lesions. Since root lesions were present at the affected level of the lumbar cord, it seems justifiable to conclude that the cord was invaded along the nerves from the inoculated site at least 10 to 14 days prior to the time the animal was examined and that the virus did not spread further. In the second monkey the process was also chronic and most marked in the lumbar cord but a few very small focal lesions were also present in some sections of the thoracic and cervical cord, medulla, midbrain, and thalamus; many intervening levels of spinal cord showed no lesions at all and the impression was gained that extension beyond the point of entry in the lumbar cord was along special neuronal pathways.

The inoculated muscles and viscera of monkeys which received the kidney passage 51 virus were not examined histologically. Such examinations were made, however, in 5 monkeys which exhibited no paralysis after intramuscular injection of $10^7$ $TCD_{50}$ of kidney passage 60 virus and no significant lesions were found. The occasional minor focal mononuclear infiltrations mentioned in the description of the monkeys inoculated with Mahoney virus were also found here, and were also present in the muscles and viscera of another group of 5 monkeys which were inoculated with only 10 $TCD_{50}$ of the same culture fluid and remained uninfected as indicated by absence of development of antibody.

*Observations on Infection by the Oral Route.*—The Y-SK virus produces a high incidence of paralysis in orally infected *cynomolgus* monkeys, but, contrary to the observations made with the Mahoney virus, the proportion of inapparent (antibody-producing) infection rises as the dose of virus ingested is diminished (13). Only the kidney passage 10 tissue culture fluid was tested by the oral route in 20 *cynomolgus* monkeys, and it was found to have as high a paralytogenic capacity (61 per cent paralysis; 78 per cent with CNS lesions) as the original *cynomolgus* CNS suspension.

*Effect of Different Methods of Propagation on Cytopathogenic Effects on Fibroblasts.*—It has previously been reported (7) that while Y-SK virus that is propagated by intracerebral passage in mice yields a cytopathogenic titer for *cynomolgus* testicular fibroblasts which is only slightly lower than that for renal epithelial cells, it takes only 2 intracerebral passages in *cynomolgus* monkeys to eliminate completely the cytopathogenic effect on fibroblasts without in any way altering the marked activity against renal epithelial cells. After the 10 terminal dilution passages in kidney cultures, the *cynomolgus* CNS-passaged virus produced an incomplete and questionable change in fibroblast cultures, but, following a series of passages with large inocula, the kidney passage 30 culture fluid again exhibited a high cytopathogenic titer for fibroblasts. While the kidney passage 51 culture fluid also had a high cytopathogenic titer, 7 to 12 days were required for the appearance of extensive changes even in the tubes inoculated with the largest amounts of virus.

*Effect of Different Methods of Propagation on Virulence of Y-SK Virus in Mice.*—It has already been reported (7) that while Y-SK virus, propagated by intracerebral passage in mice, possesses approximately equivalent pathogenic activity when titered intracerebrally in *cynomolgus* monkeys and mice, it takes only 2 passages in *cynomolgus* brain to suppress very markedly the number of virus particles which are pathogenic for mice by the intracerebral route. It should be noted that, after appropriate corrections for the different volumes employed in the tests on the virus in the *cynomolgus* CNS suspension, the 50 per cent end-point by intracerebral titration in mice was only $\frac{1}{200}$ of that obtained in the intracerebral titration in *cynomolgus* monkeys and only $\frac{1}{800}$ of that obtained in the test for cytopathogenic activity for renal epithelial cells (Table IX). Virus propagated in mice has almost the same activity by intracerebral titration in mice and by cytopathogenic titer in *cynomolgus* renal epithelial cells (7). It is, therefore, of special interest that, after the serial propagation with progeny of single or small numbers of virus particles, the kidney passage 10 tissue culture fluid had a 50,000 times higher titer in kidney tissue culture than by intracerebral titration in mice. Actually the $10^{1.5}$ mouse $PD_{50}$ per ml. of culture fluid indicated in Table IX means that approximately 50 per cent of the mice inoculated with 0.03 ml. of undiluted culture fluid succumbed while none of those inoculated with the higher dilutions developed obvious manifestations of infection. Subsequent failure to

resist an intracerebral challenge injection of 100 $LD_{50}$ of mouse-propagated virus provided no evidence of inapparent infection in mice which survived the original inoculation of culture fluid.

There was something unusual, however, about the few mice which did succumb after intracerebral injection of large amounts of virus contained in the kidney passage 10 culture fluid—there was evidence of viral multiplication in the spinal cord by tests for cytopathogenic activity on *cynomolgus* renal epithelial cells, and virus was demonstrable by intracerebral inoculation in *cynomolgus* monkeys, but *none* was found by intracerebral passage in mice. Further passages in kidney tissue cultures, regardless of the rapidity of transfers or the terminal dilution purifications, failed to abolish the capacity of large amounts of virus to produce poliomyelitic paralysis with fatal outcomes in a small proportion of the intracerebrally inoculated mice. The value of 1 $PD_{50}$ or less per ml. of culture fluid of kidney passages 30 to 51, indicated in Table IX, is only a rough approximation, because only 10 to 25 per cent of the mice receiving 0.03 ml. of the undiluted culture fluids succumbed. The pattern, however, was the same in all the succumbing mice which were submitted to study (Table XII). Typical lesions, characterized by acute necrosis of nerve cells, neuronophagia, interstitial and perivascular infiltration, were found predominantly in the cervical cord; there was evidence of viral multiplication as measured by the cytopathogenic test in *cynomolgus* renal epithelial cells; but intracerebral passage in other mice was either negative or, if an occasional mouse succumbed, further intracerebral passage to other mice was negative.

There came to mind the observations with the viruses of Newcastle disease and influenza which had been interpreted as indicating that large amounts of virus may produce lung lesions in mice without actual multiplication (14, 15) and the observations with influenza virus in intracerebrally inoculated mice which had been interpreted as being due to the formation of "incomplete" virus because hemagglutinins and complement-fixing antigen developed in the absence of transmissible infection (16). One may recall, however, the observations of Li and Habel (17) and Li and Schaeffer (6) that in the case of Type 3 and Type 1 poliomyelitis viruses there are variants which will infect mice after intraspinal inoculation but not after intracerebral inoculation. This can be interpreted as meaning that a highly specialized type of virus particle can multiply only in certain nerve cells (presumably the motor neurones) of the spinal cord but not in other nerve cells predominantly present in the brain stem or elsewhere in the CNS, and that the usual intracerebral inoculation in mice does not deliver enough virus to the susceptible cells in the spinal cord without prior multiplication in the higher centers. It has been noted recently (18) that with more potent preparations of mouse-passaged Leon virus, a small proportion of mice also succumb after intracerebral injection.

It appeared desirable to test the hypothesis that the Y-SK virus propagated in the kidney tissue cultures was a special variant capable of attacking the motor neurones in the spinal cord but not the nerve cells predominating in the higher centers. If this were the case it should be possible to obtain positive serial passage in mice inoculated intraspinally with material in which there was no demonstrable viral multiplication by intracerebral passage. The last column in Table XII shows the results of such tests, and while the number of mice which succumbed on first spinal passage was usually not very large, further subinoculations from the spinally inoculated mice left no doubt that a virus incapable of intracerebral passage was multiplying in the spinal cord. Further proof that this is indeed the correct explanation for the observed phenomena is presented in Table XIII which shows the results of comparative intracerebral

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## TABLE XII

*Observations on Occasional Mice Which Developed Paralysis after Intracerebral Inoculation of Large Amounts of Y-SK Virus Propagated in cynomolgus Kidney Tissue Cultures*

| Original inoculum and $TCD_{50}$/0.03 ml. | Mouse No. | Time after inoculation paralysis observed and sacrificed | Site of paralysis | Lesions in | | | | Cytopathogenic titer in *cynomolgus* renal epithelium | Result of intracerebral passage in mice | Result of intraspinal passage in mice |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Cervical cord | Lumbar cord | Medulla | Remainder of CNS | | | |
| | | *days* | | | | | | *$TCD_{50}$/gm.* | | |
| Kidney passage 10 $10^{4.5}$ | 1 | 3 (dead) | Not seen | — | — | — | — | — | 0/6 | — |
| | 2 and 3 | 2 | LF and RF | — | — | — | — | $10^{6.2}$ | 0/19* | — |
| Kidney passage 30 $10^{6}$ | 1 | 3 | RF | + | 0 | 0 | 0 | $10^{7.0}$ | 0/10 | 2‡/10 |
| | 2 | 7 | RF | + | 0 | 0 | 0 | $10^{6.7}$ | 1§/9 | 6‖/10 |
| | 3 | 23 | RF, RR, LR | + | 0 (low) | + | — | $10^{6.7}$ | 0/9 | — |
| Kidney passage 34 $10^{5.4}$ | 1 | 7 | RR | 0 | 0 | — | — | $10^{6.7}$ | 0/9 | 3¶/10 |
| Kidney passage 51 $10^{5.4}$ | 1 | 2 (dead) | Not seen | + | 0 | — | — | $10^{6.2}$ | 1/9 | — |
| | 2 | 2 (dead) | Not seen | — | 0 | — | — | $10^{6.7}$ | 1/10 | — |
| | 3 | 3 | RF, LF | + | 0 | 0 | 0 | $10^{6.2}$ | 0/9 | 2**/8 |
| | 4 | 10 | RR | 0 | + | 0 | 0 | $10^{4.2}$ | 0/10 | 2/10 |

RF = right front; LF = left front; RR = right rear; LR = left rear.

* Complete titration in range of $10^{-1}$ to $10^{-6}$ was negative in mice, which also failed to develop immunity to a subsequent intracerebral challenge with 100 $LD_{50}$ of Lansing virus. Intracerebral injection of 1 ml. of 1:20 dilution of material, which was negative in mice, into 4 *cynomolgus* monkeys produced paralysis in 3.

§ Intracerebral subinoculation of a suspension of the spinal cord and brainstem of this mouse into 10 other mice was negative.

‡ Intracerebral passage of material from 1 mouse was negative in mice (0/10), while intraspinal passage was positive (9/10).

‖ Material from 2 of these mice was subinoculated into other mice with the following results: Mouse 1—intracerebral 2/10, intraspinal 10/10; Mouse 2—intracerebral 0/10, intraspinal 4/10.

¶ Material from 1 of these mice was subinoculated into other mice with the following result: intracerebral 0/10; intraspinal 8/10.

** Material from 1 of these mice was subinoculated into other mice with the following result: intracerebral 0/10; intraspinal 8/10.

## TABLE XIII

*Comparative Paralytogenic Activity of Y-SK Virus Propagated in cynomolgus Kidney Tissue Cultures after Intracerebral and Intraspinal Injection in Mice*

| Dilution of culture fluid | Kidney passage 30 | | | Kidney passage 51 | | |
|---|---|---|---|---|---|---|
| | $TCD_{50}$/0.01 ml. log | Intracerebral 0.03 ml. | Intraspinal 0.02 ml. | $TCD_{50}$/0.01 ml. log | Intracerebral 0.03 ml. | Intraspinal 0.02 ml. |
| Undiluted | 5.9 | 4/16 | 10/10 | 4.9 | 8/30 | 10/10 |
| $10^{-1}$ | 4.9 | 0/9 | 9/10 | 3.9 | 1?/19 | 10/10 |
| $10^{-2}$ | 3.9 | 0/10 | 10/10 | 2.9 | 0/19 | 8/10 |
| $10^{-3}$ | 2.9 | 0/10 | 5/9 | 1.9 | — | 1/10 |
| $10^{-4}$ | 1.9 | 0/8 | 1/10 | 0.9 | — | 0/9 |
| $10^{-5}$ | 0.9 | 0/10 | 1*/10 | 0.09 | — | 0/7 |

* Specific neuronal lesions were demonstrated in spinal cord of this dead mouse, indicating that even a very small number of $TCD_{50}$ may initiate progressive infection in the spinal cord if by chance they happen to come in contact with the special susceptible cells. The failure of 100 to 200 $TCD_{50}$ to produce any effect in the majority of intraspinally inoculated mice suggests that even a direct spinal inoculation does not insure delivery of virus to the ***specially susceptible cells***, until the number of virus particles exceeds a certain minimum.

and intraspinal titrations of kidney passage 30 and kidney passage 51 tissue culture fluids in mice. Subpassages of spinal cord suspensions from several mice in each of the intraspinal series clearly showed that serial passage was possible only by the spinal route, and that the virus multiplying in the spinal cord of the mice produced cytopathogenic titers in *cynomolgus* renal epithelium of the same order of magnitude recorded in Table XII. A neutralization test on virus recovered in tissue culture after 3 passages in the spinal cord of mice proved its antigenic identity with Type 2 poliomyelitis virus.

TABLE XIV

*Pathogenic and Immunogenic Activity of Type 3 Poliomyelitis Virus (Leon Strain) in cynomolgus Monkeys after Propagation in cynomolgus Kidney Tissue Culture. 30 rapid passages using large inocula, followed by 3 terminal dilution passages, followed by 1 passage, using large inoculum of progeny of 3rd terminal dilution*

| Dilution of culture fluid | 0.2 ml. in kidney culture | 1 ml. intracerebrally | | | 1 ml. intramuscularly | | |
|---|---|---|---|---|---|---|---|
| | Cytopathogenic effect | Paralysis | CNS lesions | Antibody | Paralysis | CNS lesions | Antibody |
| Undiluted | 4/4 | 0/4 | 0/4 | 2/3 | 0/5 | 0/5 | 5/5 |
| $10^{-1}$ | 4/4 | 0/4 | 0/4 | 0/4 | 0/5 | 0/5 | 4/5 |
| $10^{-2}$ | 4/4 | 0/4 | 0/4 | 0/4 | 0/5 | 0/5 | 2/5 |
| $10^{-3}$ | 4/4 | 0/4 | 0/4 | 0/4 | 0/5 | 0/5 | 2/5 |
| $10^{-4}$ | 4/4 | 0/4 | 0/4 | 0/4 | 0/5 | 0/5 | 0/5 |
| $10^{-5}$ | 4/4 | 0/4 | 0/4 | 0/4 | 0/5 | 0/5 | 1/5 |
| $10^{-6}$ | 4/4 | 0/4 | 0/4 | 0/4 | 0/5 | 0/5 | 0/5 |
| $10^{-7}$ | 0/4 | — | — | — | 0/5 | 0/5 | 0/3 |
| 50 per cent titer per 1 ml..... | $10^{7.2}$ | 0 | 0 | Undiluted | 0 | 0 | Irregular to $10^{-5}$ |

### *Experiments with Type 3 Poliomyelitis Virus (Leon Strain)*

No systematic study of the pathogenic and immunogenic spectra was made with this strain of virus. It seemed important to determine first of all whether or not the procedures which yielded avirulent variants of the Types 1 and 2 poliomyelitis viruses were generally applicable and would yield a similar variant of a Type 3 virus.

Accordingly the Leon strain which was being used in the laboratory for neutralization tests was submitted to an arbitrary series of rapid passages at approximately 24 hour intervals in kidney tissue cultures, followed by several terminal dilution purifications, prior to preparation of a large pool for virulence tests. The starting virus, which had 8 passages in *rhesus* testis cultures and 3 passages in *cynomolgus* kidney tissue cultures, produced prostrating paralysis in 4 to 6 days in each of 4 intracerebrally inoculated *cynomolgus* monkeys. The tests for virulence and immunogenic capacity of the "purified" kidney passage 34 tissue culture fluid in intracerebrally or intramuscularly inoculated *cynomolgus* monkeys are summarized in Table XIV. None of the 28 monkeys inoculated intracerebrally with $10^{1.2}$ to $^{7.2}$ $TCD_{50}$ of virus and none of the 40 monkeys inoculated into the leg muscles with $10^{0.2}$ to $10^{7.2}$ $TCD_{50}$ of virus developed either paralysis or demonstrable lesions in the CNS, despite

the fact that more than the usual number of levels of spinal cord and higher centers were examined histologically. The failure of any of the 24 monkeys which received $10^{6.2}$ $TCD_{50}$ or less intracerebrally to develop antibodies suggests that the virus actually did not multiply after intracerebral injection. The development of antibody by monkeys inoculated intramuscularly with $10^{4.2}$ $TCD_{50}$ and in one instance even after $10^{2.2}$ $TCD_{50}$ indicates that the virus multiplied in extraneural sites. The fact that only 80 per cent of those receiving $10^{6.2}$ $TCD_{50}$ and 40 per cent of those inoculated with $10^{5.2}$ $TCD_{50}$ developed antibody, suggests that not all *cynomolgus* monkeys are susceptible to infection with this variant. Although no histologic examination was made of the inoculated muscles and viscera of monkeys inoculated with kidney passage 34 virus, no significant lesions were found in 4 monkeys which remained well after intramuscular injection of $10^6$ $TCD_{50}$ of kidney passage 60 tissue culture fluid; one of the 4 monkeys presented a more extensive localized lesion in the inoculated muscle than was observed in controls or in any of the monkeys previously described but whether this was a specific lesion or the result of more extensive inoculation trauma could not be determined.

Preliminary studies in mice revealed the interesting fact that while the starting virus (8 passages in *rhesus* testis and 3 passages in *cynomolgus* kidney cultures) was pathogenic for mice by the spinal but not the intracerebral routes, the kidney passage 34 virus was neither paralytogenic nor otherwise virulent by either route.

## DISCUSSION

The results of the present study have demonstrated a procedure by which it has proved possible to "convert" highly virulent strains of each of the 3 immunologic types of poliomyelitis virus into avirulent variants as judged by intracerebral or extraneural inoculation of *cynomolgus* monkeys. This was achieved by manipulating multiplication of the viruses in the tissues of a single host. The data show that mere multiplication in non-nervous tissue (cultures of kidney) of the *cynomolgus* monkey had no effect on the virulence of the viruses for *cynomolgus* monkeys by the intracerebral or various extraneural routes, as long as single or small numbers of virus particles were used to initiate the cultures and a large number of multiplication cycles were permitted so that any hypothetical slow growing particles could catch up in numbers with faster growing variants. The use of large inocula ($10^5$ to $10^6$ $TCD_{50}$) to initiate the cultures together with rapid passage,—which would favor the overgrowth of particles capable of most rapid reproduction in non-nervous tissue,—led to the development of culture fluids with diminished virulence and unusual patterns of reaction in *cynomolgus* monkeys. Although purification by the terminal dilution technique did not invariably segregate the modified variants (an occurrence depending perhaps on the proportion of the different components in a culture fluid), it did ultimately yield variants which can be called avirulent for monkeys, within the defined limits, from each of the 3 immunological types. The results obtained on further cultivation of the modified Mahoney variant, to be reported in a subsequent communication, indicate that under certain conditions continued rapid passages in the same non-nervous tissue cultures can lead to the appearance of new variants which are virulent for *cynomolgus* monkeys by both intracerebral and intramuscular

routes. This observation is reminiscent of the experience with the 17 D variant of yellow fever virus in which continued serial propagation in chick embryos led in one instance to the appearance of increased encephalitogenic activity for monkeys (and man) and in another to loss of immunogenic activity (19). Further cultivation of the modified Y-SK and Leon viruses has not yielded (at least as yet) any new virulent mutants.

Although it is quite clear that the experimentally produced variants described in this communication are very different from the virulent parent strains, the word "avirulent" can be applied to them only with certain reservations and qualifications regarding the host and route of inoculation. In general a poliomyelitis virus can be regarded as virulent when it produces paralysis and as avirulent when it does not and is harmless in other respects. A poliomyelitis virus may fail to produce paralysis for any of 3 distinct reasons: (*a*) it may lack the capacity to damage lower motor neurones or even to multiply in them; (*b*) it may produce typical lesions in the motor neurones it reaches but for various reasons may not be able to spread sufficiently to affect the large number of these which must be destroyed to produce obviously apparent paralysis; (*c*) it may be unable to reach the motor neurones after extraneural or even after intracerebral inoculation. All 3 variants described in the present communication failed to produce CNS lesions in the intracerebrally inoculated monkeys; but neuronal lesions in the spinal cord, not sufficiently extensive to produce paralysis, were found in 3 of 48 monkeys which received the Mahoney strain and 2 of 20 monkeys which received the Y-SK strain intramuscularly. The fact that intracerebral passage of the virus found in the spinal cord of one of these monkeys yielded negative results on inoculation though a titer of $10^{4.7}$ $TCD_{50}$ per gram was obtained in kidney cultures, strongly suggests that these modified strains may be "spinal variants" for the monkey. Accordingly, until tests have been performed on the effect of direct spinal inoculation, one cannot be certain that the experimentally produced variants are completely avirulent for *cynomolgus* monkeys.

The pathogenicity of the experimentally developed variants for non-nervous tissue of *cynomolgus* monkeys *in vitro* and *in vivo* also requires special consideration. *In vitro*, the modified viruses destroyed not only epithelial cells growing out of kidney tissue, and fibroblasts growing out of testicular, but also appeared to have a disintegrative effect on the original pieces of tissue planted in the culture tubes. It is especially noteworthy, therefore, that in none of 14 *cynomolgus* monkeys which received approximately $10^7$ $TCD_{50}$ of the modified viruses intramuscularly, were any significant lesions found in the muscles, kidneys, testes, ovaries, heart, pancreas, adrenals, liver, and spleen. It is furthermore of interest that the modified viruses were less effective than the virulent parent viruses in initiating infection by the intramuscular and oral routes. Thus, while intramuscular injection of 10 $TCD_{50}$ of the parent Mahoney virus produced antibody in all monkeys and paralysis in most, 100,000

$TCD_{50}$ of the modified variant were required to produce antibody in all monkeys, although in an occasional animal even 10 $TCD_{50}$ sufficed. A very similar ratio was found to obtain for the oral route: thus, feeding of approximately 10,000 $TCD_{50}$ of the virulent Mahoney virus produced infection in 7 of 20 *cynomolgus* monkeys (5 paralyzed), while about 10,000 times as much of the modified virus produced infection (antibody) in only 8 of 20 *cynomolgus* monkeys (none paralyzed) (Table VI). A marked diminution in the intramuscular and oral infective capacity for *cynomolgus* monkeys of Brunhilde virus propagated in human tissue cultures has already been noted in earlier work in this laboratory, but it was also found that even smaller doses of the same virus preparation were infective (immunogenically) by the same routes in chimpanzees (20). The fact that as little as 10 $TCD_{50}$ of modified virus may produce antibody in an occasional *cynomolgus* monkey points to the necessity of determining whether such minute amounts of virus might be regularly effective in chimpanzees and human beings, who are more susceptible to infection but less susceptible to paralysis, than are monkeys.

Another observation that is worthy of special note is that about 50 per cent of the *cynomolgus* monkeys which developed antibody after ingestion of the modified Mahoney virus exhibited very much lower titers than are found in monkeys with inapparent infection after ingestion of the virulent parent strain (see Table VII). Furthermore, the monkeys which had antibody titers of less than 1:32 developed a 4- to 18-fold rise in titer after ingesting a large amount of virus of the virulent parent strain, suggesting that they were infected inapparently a second time, while the animals with initial titers of 1:32 or more showed no significant change in antibody level after swallowing the virulent virus.

Pathogenicity for mice, even of the limited type obtaining only after spinal inoculation, does not appear to be linked in any direct or constant manner to pathogenicity for *cynomolgus* monkeys. The Type 1 (Mahoney) and Type 2 (Y-SK) variants produced experimentally are paralytogenic in intraspinally inoculated mice while the Type 3 (Leon) is not. In the Type 1 virus, the spinal mouse-pathogenic property appeared when the intracerebral monkey-pathogenic property disappeared. In the case of the Type 2 virus on the other hand, the intracerebral mouse-pathogenic property disappeared after the first series of terminal dilution cultivations in *cynomolgus* kidney tissue whereas the spinal mouse-pathogenic property and full intracerebral virulence for monkeys remained associated throughout a long series of subsequent cultures; but when at last the intracerebral monkey-pathogenic property disappeared the spinal mouse-pathogenic property still remained. Yet another pattern obtained for the Type 3 virus, where both the spinal mouse-pathogenic and intracerebral monkey-pathogenic properties possessed by the parent strain were absent in the final variant produced experimentally.

SUMMARY

Attempts were made to "convert" highly virulent strains of the 3 immunologic types of poliomyelitis virus (Mahoney, Y-SK, and Leon) into avirulent variants. Tests involving intracerebral, intramuscular, or oral administration of virus to *cynomolgus* monkeys indicated that mere propagation in cultures of kidney tissue of *cynomolgus* monkeys had no effect on virulence when single or small numbers of virus particles were used as seed, and harvests were delayed for 24 hours or more after the appearance of cytopathogenic change. On the other hand, passages at 24 hour intervals with large inocula ($10^5$ to $10^6$ $TCD_{50}$) produced culture fluids with diminished virulence and unusual patterns of response in *cynomolgus* monkeys. Purification of such culture fluids by the terminal dilution technique yielded modified strains which proved to be avirulent after administration by the intracerebral, intramuscular, or oral routes in *cynomolgus* monkeys.

Neither paralysis nor CNS lesions were found in any of more than 80 monkeys inoculated intracerebrally with various amounts of virus. However, focal neuronal lesions were found in the spinal cord of 3 of 48 monkeys inoculated intramuscularly with various amounts of the Mahoney variant, in 2 of 20 receiving the Y-SK variant, though in none of 40 inoculated with various amounts of the Leon variant. Virus recovered from the spinal cord of one of the monkeys in the Mahoney group produced no paralysis on intracerebral passage in monkeys. It is assumed that all 3 modified viruses possess a limited capacity to affect lower motor neurones of *cynomolgus* monkeys when these are directly exposed to them by accidental intraneural or traumatic intracerebral injection.

On propagation in *cynomolgus* kidney cultures the modified viruses reached titers of approximately $10^7$ $TCD_{50}$ per ml., as measured by cytopathogenic activity on renal epithelial cells *in vitro*, yet produced no perceptible pathologic changes in the muscles, kidneys, testes, ovaries, heart, pancreas, adrenals, liver, or spleen of *cynomolgus* monkeys inoculated intramuscularly. The modified viruses were immunogenic after intramuscular injection, but a large proportion of *cynomolgus* monkeys failed to develop antibody after small doses, indicating that in this host the experimentally produced variants multiplied less readily in non-nervous tissue than the virulent parent strains. Tests with the Type 1 virus showed that the orally administered avirulent variant can induce the formation of antibody and bring about resistance to the occurrence of paralysis such as results from ingestion of the virulent, parent strain.

The Types 1 and 2 modified viruses are paralytogenic in mice after direct spinal inoculation whereas the Type 3 virus is not. The Type 1 virus became paralytogenic for mice when it lost its virulence for *cynomolgus* monkeys by the indicated routes. The Type 2 virus lost its virulence for mice by the in-

tracerebral but not intraspinal routes when it was still fully virulent for *cynomolgus* monkeys, and retained its paralytogenic activity in intraspinally inoculated mice after it had lost its virulence for *cynomolgus* monkeys by the indicated routes. The parent Type 3 virus was paralytogenic in intraspinally inoculated mice when it was still fully virulent for *cynomolgus* monkeys, but this property disappeared in the modified virus when it became avirulent for monkeys.

*Addendum.*—Tests performed after this manuscript was submitted for publication indicated that all 3 experimentally produced variants, which are avirulent for *cynomolgus* monkeys after intracerebral, intramuscular, or oral administration, can produce localized paralysis after intraspinal inoculation. However, all 3 viruses failed to produce paralysis or lesions after spinal injection in 9 chimpanzees (3 for each immunologic type). The details of these observations will be reported in a forthcoming communication.

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